# New Understanding of β-Cell Heterogeneity and In Situ Islet Function

Benninger, Richard; Hodson, David

*DOI:*
10.2337/dbi17-0040



*Document Version*
Peer reviewed version

*Citation for published version (Harvard):*
Benninger, R & Hodson, D 2018, 'New Understanding of β-Cell Heterogeneity and In Situ Islet Function', *Diabetes*, pp. 537-547. https://doi.org/10.2337/dbi17-0040

American Diabetes Association. diabetes

# New understanding of beta cell heterogeneity and in situ islet function

| | |
|---|---|
| Journal: | *Diabetes* |
| Manuscript ID | DBi17-0040.R1 |
| Manuscript Type: | Perspectives in Diabetes (invited or by proposal) |
| Date Submitted by the Author: | 07-Dec-2017 |
| Complete List of Authors: | Benninger, Richard; University of Colorado at Denver - Anschutz Medical Campus, Barbara Davis center for childhood diabetes<br>Hodson, David; University of Birmingham, Institute of Metabolism and Systems Research (IMSR) |
| | |

# New understanding of beta cell heterogeneity and in-situ islet function

**Richard KP Benninger[a,b] and David J Hodson[c,d].**

[a] Department of Bioengineering, University of Colorado Anschutz Medical Campus, Aurora, CO. 80045.

[b] Barbara Davis Center for Diabetes, University of Colorado Anschutz Medical Campus, Aurora, CO. 80045.

[c] Institute of Metabolism and Systems Research (IMSR), University of Birmingham, Edgbaston, B15 2TT, UK.

[d] Centre for Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, B15 2TH, UK; and COMPARE University of Birmingham and University of Nottingham Midlands.

To whom correspondence should be addressed:

richard.benninger@ucdenver.edu Tel: +1 (303) 724-6388.

1775 Aurora court, Barbara Davis Center for Childhood Diabetes, University of Colorado Anschutz Medical campus, Aurora, CO. 80045.

d.hodson@bham.ac.uk Tel: +44 (0)121 414 6896

Office 227, IBR Tower, Institute of Metabolism and Systems Research, University of Birmingham, Edgbaston, B15 2TT, UK

Word count: 4560

Key words: heterogeneity, insulin secretion, stimulus-secretion coupling, intercellular communication, electrical stimulation, optogenetics

**ABSTRACT:**

Insulin secreting β-cells are heterogeneous in their regulation of hormone release. While long known, recent technological advances and new markers have allowed the identification of novel subpopulations, improving our understanding of the molecular basis for heterogeneity. This includes specific sub-populations with distinct functional characteristics, developmental programs, abilities to proliferate in response to metabolic or developmental cues, and resistance to immune-mediated damage. Importantly, these sub-populations change in disease or in aging, including in human disease. While discovering new β-cell sub-populations has substantially advanced our understanding of islet biology, a point of caution is that these characteristics have often necessarily been identified in single β-cells dissociated from the islet. β-cells in the islet show extensive communication with each other via gap junctions, and with other cell types via diffusible chemical messengers. As such, how these different sub-populations contribute to in-situ islet function, including during plasticity, is not well understood. We will discuss recent findings revealing functional β-cell sub-populations in the intact islet, the underlying basis for these identified sub-populations, and how these sub-populations may influence in-situ islet function. Furthermore, we will discuss the outlook for emerging technologies to gain further insight into the role of sub-populations in in-situ islet function.

**Introduction to β-cell heterogeneity:**

A β-cell is a terminally differentiated cell that produces and secretes insulin in a glucose-regulated manner. Importantly, β-cells have the ability to adapt to changes in metabolic demand through increased insulin secretion and/or number. In most vertebrate species β-cells form clusters with other hormone secreting cells (glucagon-secreting α-cells, somatostatin-secreting δ-cells) within islets of Langerhans. Very early studies of the β-cell assumed them to be homogenous based on a lack of morphological differences. However, detailed studies subsequently determined that there exists a broad heterogeneity in the function of β-cells. These early studies of β-cell heterogeneity are summarized by the landmark review of Pipeleers (1), which describes with remarkable foresight the presence, characteristics and role of functional β-cell sub-populations. This includes how dissociated β-cells show functional heterogeneity, with populations of cells displaying higher levels of glucose metabolism, redox state, insulin synthesis, membrane potential, and insulin secretion; that morphological markers (nuclear size, insulin granularity) can differentiate β-cell sub-populations with differing glucose sensitivity and insulin secretion levels; that β-cells show heterogeneous expression of key proteins such as glucokinase (GCK), connexins, or insulin, including spatial variations across the islet; that β-cells with low glucose-stimulated insulin secretion preferentially increase in number under development or metabolic stress; and that β-cells vary in their sensitivity to cytotoxic agents. Despite this in-depth knowledge, there have been several gaps in our understanding that have persisted until recently:

i) What is the molecular basis for β-cell functional diversity?
ii) Which markers can be used to identify and characterize β-cell sub-populations?
iii) Does functional heterogeneity in the intact islet or pancreas mirror that observed among dissociated β-cells?
iv) What is the role of β-cell heterogeneity in islet function and glucose homeostasis, and can changes in heterogeneity contribute to diabetes?
v) Are β-cells fixed in specific functional states, or can they transition between states over time?

We will describe recent technological advances and studies that have answered some of these key questions, with a focus on understanding the consequence of heterogeneity in β-cell function within the islet setting.

**Recent advances characterizing β-cell heterogeneity:**

Early and more recent studies demonstrated heterogeneity in insulin secretion in dissociated mouse or human β-cells using the hemolytic plaque assay (2). Patch clamp measurements also revealed heterogeneity in dissociated β-cell electrical properties (3). Autofluorescence measurements revealed heterogeneity in redox state, and incorporation of radioactive tracers revealed heterogeneity in glucose metabolism and insulin biosynthesis (4). The development of fluorescent biosensors and confocal or 2-photon microscopy provided tools to further characterize β-cell functional differences. This includes precise quantification of heterogeneity in dissociated β-cell glucose metabolism and redox state (5); glucose sensitivity to $Ca^{2+}$ elevations and $Ca^{2+}$ oscillation patterns (6); and cAMP oscillation patterns (7). Recently, the application of new biomarkers or high-throughput single-cell analyses has further revealed molecular details underlying β-cell heterogeneity.

*Markers of β-cell sub-populations:* Early studies suggested insulin granularity was a morphological marker that could separate a population of β-cells with a low glucose threshold (4). More recently, several markers have been employed that reveal β-cell sub-populations with differing function. Polysialylated (PSA)-NCAM separated two populations of mouse β-cells, with one population ($\beta^{high}$) showing higher $Ca^{2+}$ and ATP elevation, insulin secretion, as well as *Gck* and *Glut2* expression (8). Insulin promoter activity (MIP-GFP fluorescence) separated three populations of β-cells, with the MIP-$GFP^{low}$ population (~10% incidence in adult) possessing low insulin expression and low granularity (9). Aguayo-Mazzucatio and coworkers subsequently showed that the MIP-$GFP^{low}$ and MIP-$GFP^{high}$ populations decreased and increased in incidence, respectively, during aging (10). Further, the MIP-$GFP^{low}$ population was not marked by increases in the aging markers IGF1R or p16Ink4a, the lack thereof indicative of newly formed cells. The authors also showed that a population of highly secreting cells is lost during aging. However, the low incidence of this population (~5%) is suggestive of a subset of MIP-$GFP^{high}$ cells. Recently, Lickert and coworkers utilized a Fltp-Venus reporter mouse to separate two populations of β-cells. Fltp-Venus positive ($Fltp^{+}$) cells showed improved insulin secretion but reduced proliferative ability (11), together with more extensive mitochondrial morphology, more oxidative metabolism, and increased insulin granularity. The genetic profile of $Fltp^{+}$ cells was also consistent with improved function (i.e. increased expression of genes for GCK, GLUT2, GLP1R, GIPR, Cx36), as well as increased

maturity. Notably, during development or upon metabolic stress the Fltp$^-$ population showed greater proliferation and number. These studies together indicate a dynamic β-cell sub-population defined by improved glucose-stimulated insulin secretion as a result of elevated glucose metabolism and insulin granule content.

Huising and coworkers employed a Ucn3 reporter mouse revealing a population of insulin$^+$ Ucn3$^-$ cells undergoing α-cell to β-cell transition at the intact islet periphery (12). These immature cells showed decreased expression of genes regulating glucose metabolism (*Gck,* TCA cycle, oxidative phosphorylation), membrane potential ($K_{ATP}$, $Ca_V$ subunits), as well as *Glp1r* and *Ins*. They also lacked both glucose influx and glucose-regulated membrane potential and $Ca^{2+}$ influx. While reminiscent of sub-populations with reduced function, this population is much lower in incidence (~2%), shows negligible glucose sensitivity, and is not differentiated by markers such as Fltp. As such, this represents a novel cell sub-population. Herold and coworkers also identified a population of β-cells in non-obese diabetic (NOD) mice characterized by reduced granularity that increased in number during autoimmune attack of the islet (13). This population also displayed reduced levels of insulin, GLUT2 and maturity markers, consistent with earlier studies (1). Thus, rarer sub-populations of β-cells do exist, although how they overlap is not known.

In human β-cells, Grompe and coworkers identified two surface markers (CD9, ST8SIA1) that separated four populations of β-cells with distinct gene expression profiles (14). ST8SIA1$^+$ cells (~15% in healthy donors) showed lower insulin secretion, lower expression of *GLUT2*, but increased expression of genes encoding $K_{ATP}$ channel subunits and other $K^+$ channels. The incidence of ST8SIA1$^+$ cells is also increased in type 2 diabetic donors.

While these markers all separate β-cell populations with distinct functional states, further less well characterized sub-populations have been identified using other markers and the reader is referred to (15). However, it is important to remember that markers are not generally associated with driving the functional state of the β-cell sub-population. For example, a Fltp deletion has minor impact on β-cell function (11). Thus, PSA-NCAM or CD9/ST8SIA1 may simply separate differing populations in an arbitrary manner. Furthermore, it is not always clear whether fluorescent reporter constructs reflect the protein level: for example, MIP-GFP$^{medium}$ and MIP-GFP$^{high}$ β-cells show identical insulin transcription and protein levels (9), and the change in *Fltp* gene transcription only varies ~2-fold between Fltp+

and Fltp- cells defined by the Fltp-Venus reporter (11). Thus, great care should be taken when linking the marker involved to the characteristics of the sub-population in question.

*High-throughput single-cell analysis:* In an elegant study, Kaestner and coworkers employed mass-cytometry to detect large numbers of protein markers in human β-cells, indicating three consistent β-cell states (C1, C2, C3) (16). One state (C1, 10-70% in adult) showed substantially lower proliferation than the other two states (C2, C3), where the proportion of C1 increased with age and decreased in obesity. While limited probes for proteins involved in β-cell function were employed, the C1 population showed higher PDX1 and insulin expression indicative of a mature functional population. The C1 population also showed lower ST8SIA1 and CD9 levels compared to proliferating C2, C3 populations; where previously ST8SIA1$^-$ and CD9$^-$ β-cells showed improved insulin secretion and maturity markers, and decreased in T2D (14).

Single-cell sequencing has been used by several groups to examine the transcriptional profile in islet endocrine cells (16-18) (for comprehensive reviews on this topic, see (15;19)). This has also resolved sub-populations of β-cells in humans (17), identifying five β-cell states. Interestingly, all showed similar insulin expression, but differed in gene transcription that included free-fatty acid receptor 4 and RBP4 (an adipokine receptor). However, it remains unclear whether these states overlap with those revealed by mass-cytometry or markers described to date. Further, no clear heterogeneity was detectable using similar analyses in mouse β-cells (20), questioning whether this may reflect true species differences, a reduction in variability due to use of syngeneic donors maintained in controlled housing conditions, or an effect of isolation protocol. Moreover, single cell analysis characterizes heterogeneity in dissociated β-cells, and islet dissociation may bias the population of cells that are analyzed, for example due to reduced β-cell viability. Integration of multiple factors (e.g. environmental stimuli, developmental cues, cell cycle and tissue architecture) that affect cell identity will also be important in future studies to robustly define sub-populations (21).

*Interpreting sub-populations from marker and single cell analyses:* Fig.1 summarizes the functional β-cell sub-populations identified by recent marker and single-cell sequencing studies and their relative overlap in characteristics. Bearing in mind potential limitations in marker studies or high throughput single cell studies, these studies consistently show a

mature population of β-cells (incidence 50-80%) that is functionally competent, probably due to higher rates of oxidative metabolism and ATP production, high levels of insulin expression, granule content and secretion, and potentially improved electrical properties. This population reduces in incidence or shows dysfunction under aging and diabetes, possibly due to increased susceptibility to ER stress (22). Conversely, the opposing less-mature population (incidence 15-50%) shows reduced insulin secretion secondary to reductions in glucose metabolism and insulin expression, but has improved proliferative capacity.

While some studies indicate that >2 populations exist (e.g. MIP-GFP$^{low/medium/high}$, CD9$^{+/-}$/ST8SIA1$^{+/-}$), it is unclear whether the 'functionally competent' and 'less functional' populations can be further subdivided. Likely, there exist small distinct sub-populations, such as the Ucn3$^{-}$ insulin$^{+}$ neogenic niche, with some similarities to the 'less functional' population. Future work will require care when determining whether identified sub-populations truly are distinct, or whether they are an artifact of the analysis employed. For example, does increasing the number of markers generate more or less apparent/overlapping β-cell populations, or can populations be reproduced in a second dataset (e.g. using training and test sets)? Can absence of a subpopulation be excluded given the relatively poor resolution of 'omics approaches such RNASeq, where even at 6,000M reads a proportion of genes remains unquantified?

Importantly, most studies to date described above have characterized sub-populations using single-cell preparations that lack the influence of the interactions between β-cells and other cell types within the islet. While re-aggregation methods can recover some of these influences, it is still unclear how different sub-populations may interact within the islet to influence islet function. Therefore, proper consideration of how a heterogeneous population of β-cells functions within the islet is needed.

**How heterogeneous cells interact in the islet:**

Interactions between cells within the islet are critical: dissociation of the islet into single β-cells reduces the dynamic range of glucose-stimulated insulin secretion by ~10-fold (23). Even when β-cells remain coupled to one or two other β- or α-cells, insulin secretion significantly improves (24). While many mechanisms of cell-cell communication have been identified, less is known about how they coordinate heterogeneous cell populations.

*Gap junction electrical coupling between heterogeneous cells:* Gap junction (GJ) channels electrically couple β-cells within mouse and human islets (25), serving two main functions. Firstly, GJ channels coordinate oscillatory dynamics in electrical activity and $Ca^{2+}$ under elevated glucose or GLP1, allowing pulsatile insulin secretion (26;27). Secondly, GJ channels lower spontaneous elevations in $Ca^{2+}$ under low glucose levels (28). GJ coupling is also heterogeneous within the islet (29) leading to some β-cells being highly coupled, yet others showing negligible coupling.

Several studies have examined how electrically heterogeneous cells interact via GJ channels using $K_{ATP}$ channel mutant mice (Fig.2). The Kir6.2$^{[\Delta N30,K185Q]}$ mouse model CreER-dependently expresses overactive $K_{ATP}$ channels in a population of β-cells, rendering them inexcitable (30). By varying recombination efficiency, as few as ~15% inexcitable β-cells was found to be sufficient to suppress $Ca^{2+}$ responses to glucose across the islet (30). Thus, small numbers of inexcitable cells can cause marked dysfunction by suppressing islet electrical activity and insulin secretion. Similar conclusions were made using the Kir6.2$^{[AAA]}$ mouse model that expresses inactive $K_{ATP}$ channels in ~70% of β-cells, rendering them hyper-excitable (31). Despite the majority of β-cells lacking glucose-regulated $K_{ATP}$ closure, islets from these mice showed near-normal responses, since the remaining ~30% of normal β-cells were sufficient to regulate islet $Ca^{2+}$ (31). In each case, a deficiency in GJ channels led to $K_{ATP}$ mutant cells and normal cells behaving distinctly (30;31). Thus, GJ coupling is a major mechanism by which electrically heterogeneous cells interact. Early studies imposing metabolic heterogeneity through a knockout of GCK in a population of β-cells did not find evidence of significant 'metabolic coupling' between β-cells (32).

This series of experiments indicate a 'bistability' in islet function, where a threshold number of poorly-responsive β-cells is sufficient to totally suppress islet function. Notably, when islets lacking GJ channels are treated with low-levels of the $K_{ATP}$ activator diazoxide or the GCK- inhibitor mannoheptulose, a sub-population of cells are silenced, presumably corresponding to the 'less functional' population (30). Only diazoxide/mannoheptulose concentrations capable of silencing >40% of these cells will fully suppress $Ca^{2+}$ elevations in normal islets. Again this indicates that a threshold number of poorly-responsive cells can inhibit the whole islet. Thus, if there exists a threshold number of functionally competent β-cells (~60-85%), then the islet will show coordinated elevations in $Ca^{2+}$ and insulin secretion. Below this threshold number, the islet will lack $Ca^{2+}$ elevation and insulin secretion (Fig.2).

The precise threshold depends on the characteristics of the excitable and inexcitable populations: small numbers of inexcitable cells will increase the number of functionally competent cells required for islet activity, whereas small numbers of highly excitable cells will do the opposite. However, if GJ coupling is lowered then inexcitable cells will exert a reduced suppression, also decreasing the threshold required.

*Paracrine and juxtacrine communication:* Paracrine communication between β-cells and other endocrine cells is also important for regulating insulin secretion. Complex cross-talk between α- and β-cells- stemming from neurotransmitters, ions and hormones- shapes glucagon and insulin secretion depending on glucose concentration (reviewed in (33)). δ-cell somatostatin secretion also inhibits insulin secretion. Notably, Ucn3 co-secreted with insulin promotes somatostatin secretion, leading to negative feedback against overt fluctuations in insulin secretion (34). Therefore, dynamic inter-communication exists between the main endocrine cells types within the islet. Several neurotransmitters (e.g. acetylcholine, glutamate, GABA) are also released within the islet by nerve terminals and endocrine cells, which can modulate insulin secretion and act on other endocrine cells (reviewed in (35)).

Juxtacrine or contact-dependent communication includes EphA-ephrinA bidirectional signaling between β-cells (36). EphA forward signaling predominates at elevated glucose and promotes insulin secretion, but ephrinA reverse signaling predominates at low glucose and suppresses insulin secretion (36). NCAM signaling between β-cells also promotes insulin secretion (8).

Little is known how these paracrine and juxtacrine mechanisms impact heterogeneous cells. One possibility is a homogenizing effect: for example, given heterogeneity some β-cells will secrete more insulin and co-secrete more Ucn3, thus stimulating more local somatostatin secretion. As a result, there may be a greater local suppression of insulin secretion creating more homogeneous release. Whether such an homogenizing effect occurs with the various paracrine communication feedback loops (e.g. somatostain-Ucn3, glucagon-insulin) remains to be determined. Similarly, juxtacrine mechanisms may impact heterogeneous cells differently, particularly give heterogeneity in β-cell glucose metabolism. However, these actions also remain to be determined.

**Heterogeneity in the intact islet:**

Functional heterogeneity in the islet, in terms of glucose metabolism (NADH response, GCK expression) (32;37), has been long known. While electrical activity and cAMP responses are apparently homogeneous in the islet (26;38), this is likely due to GJ coupling. In the absence of GJ coupling the electrical response is heterogeneous in terms of glucose sensitivities and patterns of $Ca^{2+}$ oscillations (26). Despite several aspects of function being homogeneous in the islet, β-cell sub-populations can dramatically influence overall islet function as discussed above.

*A role for existing sub-populations?:* MIP-GFP$^{high}$, PSA-NCAM$^{high}$, Fltp$^{+}$, ST8SIA1$^{-}$ 'functionally competent' β-cell sub-populations all show similar characteristics with incidences of 50-80%. Given our understanding of how heterogeneous cells interact via GJ coupling it is tempting to speculate that the opposing 'less functional' β-cell sub-population (i.e. MIP-GFP$^{low}$, PSA-NCAM$^{low}$, Fltp$^{-}$, ST8SIA1$^{+}$; 15-50%) could significantly suppress islet activity via electrical coupling. However, Fltp$^{-}$ cells showed a marked reduction in *Gjd2* expression encoding Cx36, which would be expected to reduce their ability to suppress $Ca^{2+}$ elevations across the islet, thus allowing for normal islet function. As such, the increased number of Fltp$^{-}$ cells in development or metabolic stress will likely not impair islet function. Whether MIP-GFP$^{low}$, PSA-NCAM$^{low}$, or ST8SIA1$^{+}$ cells show reduced *Gjd2*/Cx36 expression remains to be determined. Therefore, as a result of functional remodeling, the smaller 'less functional' immature β-cell population within the islet is unlikely to have a detrimental effect on overall islet function. However, until recently it has been unclear whether further sub-divisions within the 'functionally competent' sub-population have a disproportionate role within the islet, in part owing to a lack of means to discover these sub-divisions.

*Optogenetics:* Interrogation of β-cell function within islets requires technologies with high spatio-temporal resolution to capture and manipulate signaling events that occur throughout the islet. This can be achieved using optogenetics, where ion channels or pumps derived from light-sensitive bacteria are recombinantly expressed in the cell membrane to optically modulate electrical excitability and β-cell function (39). Combined with high-speed optical imaging, this provides a powerful platform for understanding sub-population function. Pioneering studies by Reinbothe and co-workers showed the utility of channelrhodopsin-2 (ChR2), a light-activated cation channel, to modulate islet $Ca^{2+}$ fluxes using a blue light (39).

Later studies used Halorhodopsin (eNpHR3.0), an orange light-activated $Cl^-$ pump, as well as ChR2 to selectively silence or activate single β-cells, as well as clusters and larger regions (40;41) (Fig.3).

*Using optogenetics to discover and understand β-cell sub-populations:* Optogenetic mapping has provided evidence that a small subset of 'functionally competent' β-cells may play a disproportionate role in orchestrating islet responses to glucose (Fig.3) (40). Random activation of single β-cells using ChR2 revealed significant elevation of $[Ca^{2+}]_i$ in neighboring regions in ~50% of cases, presumably corresponding to the 'functionally competent' β-cell sub-population. However, substantial activation was achieved in rare cases (~5%), with $Ca^{2+}$ spreading throughout large portions of the islet. Supporting an important link between glucose metabolism and heterogeneity indicated in prior studies, elevation of $Ca^{2+}$ was most effective in stimulated cells that had the highest NAD(P)H responses (41). Furthermore, a separate sub-region of β-cells was identified, characterized by low NAD(P)H responses, that showed high intrinsic oscillatory frequencies and corresponded to initiating $Ca^{2+}$ wave propagation (41). Along similar lines, targeted silencing using eNpHR3.0 highlighted a sub-population (~1-10%) of metabolically-adapted β-cells involved in supporting islet-wide $Ca^{2+}$ dynamics (40). These cells, termed 'hub cells', were typified by elevated GCK content and hyperpolarized mitochondria, highly consistent with other 'functionally competent' sub-populations. However, they also possessed ~2-5-fold lower protein expression levels of Pdx1/Nkx-6.1/insulin indicating relative immaturity, although all three markers were still detectable (Fig.1 and Fig.3). Whether eNpHR3.0-defined 'hub' and ChR2-defined subpopulations are fixed in time or dynamic remains unknown due to the inability to record for more than a few hours, although longitudinal intravital imaging techniques may aid this (42). That these more distinct populations were missed in biomarker analysis or high throughput single-cell analysis likely indicates the importance of their presence in the intact islet for function, and further argues for the importance of studying β-cell heterogeneity in the islet context.

An improvement in metabolic properties and function in hub cells is difficult to reconcile with a loss of β-cell identity. However, it is worth noting that glucose-stimulated insulin secretion in MafA deficient β-cells (i.e. less mature) is only reduced by ~50% with minimally-affected NAD(P)H responses (43), and islets deficient in GLUT2 display preserved

second phase insulin secretion (44). Moreover, recent studies have shown that immature, proliferative β-cells highly express genes involved in amino acid metabolism and mitochondrial function (45). Lastly, sub-populations of mouse β-cells exist with high *Gck,* but low *Pdx1/Nkx6-1* (20), as well as low insulin expression (46). Thus, these data indicate a sub-division within the 'functional' population in the islet, raising interesting questions about the role of β-cell identity in islet function and insulin secretion.

*Extrapolating single-cell to islet studies:* Some caution is required when trying to extrapolate results between transcriptional and functional studies. As discussed above, it is well acknowledged that tissue architecture influences gene expression and dissociated cell mRNA profiles may not necessarily reflect those in-situ (47). Isolation of single cells is usually associated with decreases in viability. While these cells are excluded from RNA-Seq analyses of purified populations, some sub-populations, including hub cells (40) or those that express low SERCA2/low Pdx1 (48), appear more susceptible to ER stress and apoptosis/cell death. As such their gene/protein expression levels may not be fully captured. Lastly, there is a tendency to classify and label sub-populations, whereas β-cells likely occupy an overlapping continuum due to the dynamic nature of gene transcription (47). Thus, a major obstacle to our present understanding of heterogeneity is the inability to track transcriptional/protein dynamics in single β-cells in-situ and over long time-scales.

*Islet heterogeneity in-vivo:* Islets are highly vascularized and pioneering intravital imaging studies in mice have shown that islet blood flow is directional, perfusing α-cells before β-cells (49). Key to understanding heterogeneity in-vivo will be experiments using islets transplanted into the anterior chamber of the eye (ACE) (42), where blood and neural supplies remain intact. Combined with optogenetics and reporter gene imaging (see below), this provides a powerful means to longitudinally image the plasticity of sub-populations across lifespan. However, the ACE is unable to recapitulate normal pancreatic physiology (e.g. endocrine-exocrine interactions), and immune infiltration has been reported (50).

**Future tools for the interrogation of β-cell heterogeneity:**

We have argued the importance for resolving and studying β-cell heterogeneity in the context of the intact islet. While we have discussed several new technologies that have been developed and applied to examine β-cell heterogeneity, additional technologies show great promise for studying β-cell heterogeneity in-situ.

*Photopharmacology:* describes the synthesis of pharmacophores bearing azobenzene photoresponsive elements whose inactive and active states can be controlled by light through *cis*- and *trans*-isomerization. These approaches have been applied to anti-diabetic agents including sulfonylureas or incretin-mimetics (51). A major advantage of photopharmacology over optogenetics is the ability to control endogenous targets without the need for recombinant approaches thus providing high-utility for targeting human β-cells. However, these approaches tend to be non-binary, with some drug activity seen even with 'inactive' or unilluminated compound, which can complicate functional interrogation of beta cell signaling.

*MALDI imaging:* Matrix-Assisted Laser Desorption Ionization (MALDI) is a label-free method that provides information on endogenous proteins, metabolites, lipids and hormones. This requires application of a matrix to allow absorption of laser energy followed by desorption and liberation of single protonated species, whose time-of-flight (TOF) is ranked according to analyte. While the spatial resolution of MALDI-TOF is low (20-200 μm), many endogenous markers can be studied simultaneously in-situ with high sensitivity. As such, it may be useful for understanding heterogeneity between individual islets or islet regions (52), although the technique is temporally limited due to the requirement to use fixed tissue.

*Multiplexed ion beam imaging:* Mass-cytometry overcomes limitations in channel number with conventional flow cytometry by using rare-earth labelled antibodies and TOF detection. This enables large (>40) protein panels to be studied and has been applied to dissociated human β-cells resolving three distinct sub-populations (16). Technology using rare-earth metal labelling and TOF detection has been adapted into an imaging format- Multiplexed Ion Beam Imaging (MIBI)- that can achieve sub-cellular spatial resolution (53). Therefore, imaging of islets in-situ, including in intact pancreas preparations will be achievable. One potential limitation will be the availability of compatible metal-labelled antibodies against proteins involved in β-cell function (e.g. GCK/ion channels/GPCRs).

*Reporter gene imaging:* Gene expression dynamics (47) may add another level of heterogeneity to β-cell function, and potentially explain some of the differences in β-cell sub-populations observed to date. Early studies by Szabat and co-workers used a Pdx1-mRFP/Ins1-GFP dual reporter to show the existence of a $Pdx1^{+}/Ins^{low}$ sub-population (46). A similar approach can be envisaged using a combination of fluorophores with long and short half-lives to allow measurement of protein expression dynamics (as an indirect readout for gene expression) in the intact islet setting, as well as examine plasticity in β-cell sub-populations. However, the disadvantage of such approaches is the inability to visualize transcription/translation of endogenous genes/RNAs, or account for posttranscriptional regulation.

*RNA FISH:* Fluorescent in-situ hybridization (FISH) allows nascent RNA to be labeled, providing a snapshot of transcription. High-throughput single-cell FISH has been applied to examine changes in mRNA levels for β-cell "repressed" genes during proliferation (54). Closer examination of the FISH data revealed marked differences between individual β-cells (10-100-fold), suggesting heterogeneity at the transcript level (54). By using higher resolution single-molecule detection (fliFISH) to robustly quantify RNA levels in pancreas tissue sections, marked heterogeneity in *Ins2* and *Nkx2-2* was observed between β-cells. This included observing more immature (low *Ins2*, *Nkx2-2*) signatures at the islet periphery (55).

*CROP- and Peturb-seq:* Differential gene expression can be obtained in single genome-edited cells residing within a pooled population by directly linking guide RNA expression with individual cell transcriptome responses. For example via incorporation of polyadenylated sequences recognized by RNASeq, or combining gRNA with expression of a bar code (56). Both techniques allow high-throughput CRISPR screening of signaling/regulatory pathways in heterogeneous populations, and could potentially be used to understand the interactions underlying heterogeneity at different developmental stages.

*Computer modeling:* To move beyond a qualitative overview (Fig.1), computer models show promise in reconciling divergent data sets generated from the range of available technologies ('omics, imaging) and sample preparations (mouse, human, intact, dissociated). Computer

models accurately described how a defined β-cell population impacts islet stimulus-secretion, e.g. $K_{ATP}$ mutant (30). Computer models also accurately described stimulus-secretion in sub-populations uncovered through ChR2-stimulation (41). We envision islets could be modelled to incorporate sub-populations of β-cells with characteristics defined by single-cell approaches, and these models compared with functional studies. Rapid in-silico screening could then identify overlap between sub-populations identified using different experimental approaches and predict how they would influence islet function. A necessary step will be validating theoretical predictions with focused experiments.

**Summary:**

Spurred on by technological advances in single-cell genomics and imaging, the renewed interest in β-cell heterogeneity has changed our view of islet development and function. Pertinently, β-cell sub-populations have been identified that provide deeper mechanistic understanding of poorly-characterized processes such as proliferation, differentiation/maturation and stimulus-secretion. Thus, β-cell heterogeneity has highlighted new facets of islet plasticity, as well as how changes in this may affect islet function under conditions associated with diabetes. However, most studies to date have investigated dissociated cells, where many transcriptomic/protein features may be altered. Moreover, while the molecular signatures of sub-populations are well characterized, how these influence protein expression and functional state are poorly understood. Key to better understanding heterogeneity will be studies in the intact tissue and even in-vivo, the integration of multiple facets of a cell's identity, and models with better fidelity for reading out gene and protein expression dynamics. Indeed, several gaps in our knowledge remain: how do we robustly define a β-cell sub-population? What is the molecular basis and functional role for heterogeneity among other endocrine cells (α-cells, δ-cells)? Are sub-populations of β-cells (or other cells) fixed into a specific functional state, or are they transitional? Recent and future technological developments should allow these questions to be addressed, and in doing so may reveal new genes and pathways that can be harnessed to improve therapy, coax β-cell regeneration, and inform de novo engineering of islets and islet transplantation.

## ACKNOWLEDGMENTS

R.K.P.B was supported by National Institutes of Health (NIH) grants R01 DK102950, R01 DK106412 and OT2 OD023852; and Juvenile Diabetes Research Foundation (JDRF) grants 5-CDA-2014-198-A-N and JDRF 1-INO-2017-435-A-N. D.J.H. was supported by a Diabetes UK R.D. Lawrence (12/0004431) Fellowship, a Diabetes UK Research Grant, a Wellcome Trust Institutional Support Award, and an MRC Project Grant (MR/N00275X/1). This work has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Starting Grant 715884 to D.J.H.).

## CONFLICT OF INTEREST

No potential conflicts of interest relevant to this article were reported.

## AUTHOR CONTRIBUTIONS

R.K.P.B and D.J.H. each contributed equally to researching literature and writing this review article.

## GUARANTOR'S STATEMENT

R.K.P.B and D.J.H are joint guarantor's of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

## REFERENCES

1. Pipeleers DG: Heterogeneity in pancreatic beta-cell population. Diabetes 1992;41:777-781
2. Salomon D, Meda P: Heterogeneity and contact-dependent regulation of hormone secretion by individual B cells. Exp Cell Res 1986;162:507-520
3. Misler S, Falke LC, Gillis K, McDaniel ML: A metabolite-regulated potassium channel in rat pancreatic B cells. Proc Natl Acad Sci U S A 1986;83:7119-7123
4. Kiekens R, In 't Veld P, Mahler T, Schuit F, Van De Winkel M, Pipeleers D: Differences in glucose recognition by individual rat pancreatic B cells are associated with intercellular differences in glucose-induced biosynthetic activity. J Clin Invest 1992;89:117-125
5. Piston DW, Knobel SM, Postic C, Shelton KD, Magnuson MA: Adenovirus-mediated knockout of a conditional glucokinase gene in isolated pancreatic islets reveals an essential role for proximal metabolic coupling events in glucose-stimulated insulin secretion. J Biol Chem 1999;274:1000-1004
6. Zhang M, Goforth P, Bertram R, Sherman A, Satin L: The Ca2+ dynamics of isolated mouse beta-cells and islets: implications for mathematical models. Biophys J 2003;84:2852-2870
7. Dyachok O, Isakov Y, Sagetorp J, Tengholm A: Oscillations of cyclic AMP in hormone-stimulated insulin-secreting beta-cells. Nature 2006;439:349-352
8. Maedler K, Karaca M, Castel J, Tourrel-Cuzin C, Brun M, Géant A, Dubois M, Catesson S, Rodriguez M, Luquet S, Cattan P, Lockhart B, Lang J, Ktorza A, Magnan C, Kargar C: Exploring Functional β-Cell Heterogeneity In Vivo Using PSA-NCAM as a Specific Marker. PLoS ONE 2009;4
9. Katsuta H, Aguayo-Mazzucato C, Katsuta R, Akashi T, Hollister-Lock J, Sharma AJ, Bonner-Weir S, Weir GC: Subpopulations of GFP-marked mouse pancreatic beta-cells differ in size, granularity, and insulin secretion. Endocrinology 2012;153:5180-5187
10. Aguayo-Mazzucato C, van Haaren M, Mruk M, Lee TB, Crawford C, Hollister-Lock J, Sullivan BA, Johnson JW, Ebrahimi A, Dreyfuss JM, Van Deursen J, Weir GC, Bonner-Weir S: β Cell Aging Markers Have Heterogeneous Distribution and Are Induced by Insulin Resistance. Cell Metab 2017;25:898-910.e895
11. Bader E, Migliorini A, Gegg M, Moruzzi N, Gerdes J, Roscioni SS, Bakhti M, Brandl E, Irmler M, Beckers J, Aichler M, Feuchtinger A, Leitzinger C, Zischka H, Wang-Sattler R, Jastroch M, Tschop M, Machicao F, Staiger H, Haring HU, Chmelova H, Chouinard JA, Oskolkov N, Korsgren O, Speier S, Lickert H: Identification of proliferative and mature beta-cells in the islets of Langerhans. Nature 2016;535:430-434
12. van der Meulen T, Mawla AM, DiGruccio MR, Adams MW, Nies V, Dolleman S, Liu S, Ackermann AM, Caceres E, Hunter AE, Kaestner KH, Donaldson CJ, Huising MO: Virgin Beta Cells Persist throughout Life at a Neogenic Niche within Pancreatic Islets. Cell Metab 2017;25:911-926 e916
13. Rui J, Deng S, Arazi A, Perdigoto AL, Liu Z, Herold KC: β Cells that Resist Immunological Attack Develop during Progression of Autoimmune Diabetes in NOD Mice. Cell Metab 2017;25:727-738
14. Dorrell C, Schug J, Canaday PS, Russ HA, Tarlow BD, Grompe MT, Horton T, Hebrok M, Streeter PR, Kaestner KH, Grompe M: Human islets contain four distinct subtypes of β cells. Nat Commun 2016;7:11756
15. Roscioni SS, Migliorini A, Gegg M, Lickert H: Impact of islet architecture on beta-cell heterogeneity, plasticity and function. Nat Rev Endocrinol 2016;12:695-709

16. Wang Yue J, Golson Maria L, Schug J, Traum D, Liu C, Vivek K, Dorrell C, Naji A, Powers Alvin C, Chang K-M, Grompe M, Kaestner Klaus H: Single-Cell Mass Cytometry Analysis of the Human Endocrine Pancreas. Cell Metab 2016;24:616-626
17. Segerstolpe Å, Palasantza A, Eliasson P, Andersson E-M, Andréasson A-C, Sun X, Picelli S, Sabirsh A, Clausen M, Bjursell MK, Smith David M, Kasper M, Ämmälä C, Sandberg R: Single-Cell Transcriptome Profiling of Human Pancreatic Islets in Health and Type 2 Diabetes. Cell Metab 2016;24:593-607
18. Xin Y, Kim J, Okamoto H, Ni M, Wei Y, Adler C, Murphy AJ, Yancopoulos GD, Lin C, Gromada J: RNA Sequencing of Single Human Islet Cells Reveals Type 2 Diabetes Genes. Cell Metab 2016;24:608-615
19. Gutierrez GD, Gromada J, Sussel L: Heterogeneity of the Pancreatic Beta Cell. Front Genet 2017;8:22
20. Xin Y, Kim J, Ni M, Wei Y, Okamoto H, Lee J, Adler C, Cavino K, Murphy AJ, Yancopoulos GD, Lin HC, Gromada J: Use of the Fluidigm C1 platform for RNA sequencing of single mouse pancreatic islet cells. Proceedings of the National Academy of Sciences 2016;113:3293-3298
21. Wagner A, Regev A, Yosef N: Revealing the vectors of cellular identity with single-cell genomics. Nat Biotechnol 2016;34:1145-1160
22. Szabat M, Page Melissa M, Panzhinskiy E, Skovsø S, Mojibian M, Fernandez-Tajes J, Bruin Jennifer E, Bround Michael J, Lee Jason TC, Xu Eric E, Taghizadeh F, O'Dwyer S, van de Bunt M, Moon K-M, Sinha S, Han J, Fan Y, Lynn Francis C, Trucco M, Borchers Christoph H, Foster Leonard J, Nislow C, Kieffer Timothy J, Johnson James D: Reduced Insulin Production Relieves Endoplasmic Reticulum Stress and Induces β Cell Proliferation. Cell Metab 2016;23:179-193
23. Lernmark A: The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. Diabetologia 1974;10:431-438
24. Wojtusciszyn A, Armanet M, Morel P, Berney T, Bosco D: Insulin secretion from human beta cells is heterogeneous and dependent on cell-to-cell contacts. Diabetologia 2008;51:1843-1852
25. Cigliola V, Chellakudam V, Arabieter W, Meda P: Connexins and β-cell functions. Diabetes Res Clin Pract 2013;99:250-259
26. Benninger RK, Zhang M, Head WS, Satin LS, Piston DW: Gap junction coupling and calcium waves in the pancreatic islet. Biophys J 2008;95:5048-5061
27. Hodson DJ, Mitchell RK, Bellomo EA, Sun G, Vinet L, Meda P, Li D, Li WH, Bugliani M, Marchetti P, Bosco D, Piemonti L, Johnson P, Hughes SJ, Rutter GA: Lipotoxicity disrupts incretin-regulated human beta cell connectivity. J Clin Invest 2013;123:4182-4194
28. Benninger RK, Head WS, Zhang M, Satin LS, Piston DW: Gap junctions and other mechanisms of cell-cell communication regulate basal insulin secretion in the pancreatic islet. J Physiol 2011;589:5453-5466
29. Farnsworth NL, Hemmati A, Pozzoli M, Benninger RK: Fluorescence recovery after photobleaching reveals regulation and distribution of Cx36 gap junction coupling within mouse islets of langerhans. J Physiol 2014;592:4431-4446
30. Hraha TH, Westacott MJ, Pozzoli M, Notary AM, McClatchey PM, Benninger RK: Phase transitions in the multi-cellular regulatory behavior of pancreatic islet excitability. PLoS Comput Biol 2014;10:e1003819
31. Rocheleau JV, Remedi MS, Granada B, Head WS, Koster JC, Nichols CG, Piston DW: Critical role of gap junction coupled KATP channel activity for regulated insulin secretion. PLoS Biol 2006;4:e26

32. Piston DW, Knobel SM, Postic C, Shelton KD, Magnuson MA: Adenovirus-mediated Knockout of a Conditional Glucokinase Gene in Isolated Pancreatic Islets Reveals an Essential Role for Proximal Metabolic Coupling Events in Glucose-stimulated Insulin Secretion. J Biol Chem 1999;274:1000-1004
33. Gaisano HY, MacDonald PE, Vranic M: Glucagon secretion and signaling in the development of diabetes. Frontiers in Physiology 2012;3
34. van der Meulen T, Donaldson CJ, Caceres E, Hunter AE, Cowing-Zitron C, Pound LD, Adams MW, Zembrzycki A, Grove KL, Huising MO: Urocortin3 mediates somatostatin-dependent negative feedback control of insulin secretion. Nat Med 2015;21:769-776
35. Caicedo A: Paracrine and autocrine interactions in the human islet: more than meets the eye. Semin Cell Dev Biol 2013;24:11-21
36. Konstantinova I, Nikolova G, Ohara-Imaizumi M, Meda P, Kucera T, Zarbalis K, Wurst W, Nagamatsu S, Lammert E: EphA-Ephrin-A-mediated beta cell communication regulates insulin secretion from pancreatic islets. Cell 2007;129:359-370
37. Jetton TL, Magnuson MA: Heterogeneous expression of glucokinase among pancreatic beta cells. Proc Natl Acad Sci U S A 1992;89:2619-2623
38. Tian G, Sandler S, Gylfe E, Tengholm A: Glucose- and hormone-induced cAMP oscillations in alpha- and beta-cells within intact pancreatic islets. Diabetes 2011;60:1535-1543
39. Reinbothe TM, Safi F, Axelsson AS, Mollet IG, Rosengren AH: Optogenetic control of insulin secretion in intact pancreatic islets with beta-cell-specific expression of Channelrhodopsin-2. Islets 2014;6
40. Johnston Natalie R, Mitchell Ryan K, Haythorne E, Pessoa Maria P, Semplici F, Ferrer J, Piemonti L, Marchetti P, Bugliani M, Bosco D, Berishvili E, Duncanson P, Watkinson M, Broichhagen J, Trauner D, Rutter Guy A, Hodson David J: Beta Cell Hubs Dictate Pancreatic Islet Responses to Glucose. Cell Metab 2016;
41. Westacott MJ, Ludin NWF, Benninger RKP: Spatially Organized beta-Cell Subpopulations Control Electrical Dynamics across Islets of Langerhans. Biophys J 2017;113:1093-1108
42. Speier S, Nyqvist D, Cabrera O, Yu J, Molano RD, Pileggi A, Moede T, Kohler M, Wilbertz J, Leibiger B, Ricordi C, Leibiger IB, Caicedo A, Berggren PO: Noninvasive in vivo imaging of pancreatic islet cell biology. Nat Med 2008;14:574-578
43. Hang Y, Yamamoto T, Benninger RKP, Brissova M, Guo M, Bush W, Piston DW, Powers AC, Magnuson M, Thurmond DC, Stein R: The MafA Transcription Factor Becomes Essential to Islet -Cells Soon After Birth. Diabetes 2014;63:1994-2005
44. Guillam M-T, Hümmler E, Schaerer E, Wu JY, Birnbaum MJ, Beermann F, Schmidt A, Dériaz N, Thorens B: Early diabetes and abnormal postnatal pancreatic islet development in mice lacking Glut-2. Nat Genet 1997;17:327-330
45. Zeng C, Mulas F, Sui Y, Guan T, Miller N, Tan Y, Liu F, Jin W, Carrano AC, Huising MO, Shirihai OS, Yeo GW, Sander M: Pseudotemporal Ordering of Single Cells Reveals Metabolic Control of Postnatal β Cell Proliferation. Cell Metab 2017;25:1160-1175.e1111
46. Szabat M, Luciani DS, Piret JM, Johnson JD: Maturation of adult beta-cells revealed using a Pdx1/insulin dual-reporter lentivirus. Endocrinology 2009;150:1627-1635
47. Harper CV, Featherstone K, Semprini S, Friedrichsen S, McNeilly J, Paszek P, Spiller DG, McNeilly AS, Mullins JJ, Davis JR, White MR: Dynamic organisation of prolactin gene expression in living pituitary tissue. J Cell Sci 2010;123:424-430
48. Johnson JS, Kono T, Tong X, Yamamoto WR, Zarain-Herzberg A, Merrins MJ, Satin LS, Gilon P, Evans-Molina C: Pancreatic and duodenal homeobox protein 1 (Pdx-1) maintains

endoplasmic reticulum calcium levels through transcriptional regulation of sarco-endoplasmic reticulum calcium ATPase 2b (SERCA2b) in the islet beta cell. J Biol Chem 2014;289:32798-32810
49. Nyman LR, Wells KS, Head WS, McCaughey M, Ford E, Brissova M, Piston DW, Powers AC: Real-time, multidimensional in vivo imaging used to investigate blood flow in mouse pancreatic islets. J Clin Invest 2008;118:3790-3797
50. Mojibian M, Harder B, Hurlburt A, Bruin JE, Asadi A, Kieffer TJ: Implanted islets in the anterior chamber of the eye are prone to autoimmune attack in a mouse model of diabetes. Diabetologia 2013;56:2213-2221
51. Broichhagen J, Frank JA, Trauner D: A Roadmap to Success in Photopharmacology. Acc Chem Res 2015;51:6018-6021
52. Aichler M, Borgmann D, Krumsiek J, Buck A, MacDonald PE, Fox JEM, Lyon J, Light PE, Keipert S, Jastroch M, Feuchtinger A, Mueller NS, Sun N, Palmer A, Alexandrov T, Hrabe de Angelis M, Neschen S, Tschöp MH, Walch A: N-acyl Taurines and Acylcarnitines Cause an Imbalance in Insulin Synthesis and Secretion Provoking β Cell Dysfunction in Type 2 Diabetes. Cell Metab 2017;25:1334-1347.e1334
53. Angelo M, Bendall SC, Finck R, Hale MB, Hitzman C, Borowsky AD, Levenson RM, Lowe JB, Liu SD, Zhao S, Natkunam Y, Nolan GP: Multiplexed ion beam imaging of human breast tumors. Nat Med 2014;20:436-442
54. Klochendler A, Caspi I, Corem N, Moran M, Friedlich O, Elgavish S, Nevo Y, Helman A, Glaser B, Eden A, Itzkovitz S, Dor Y: The Genetic Program of Pancreatic beta-Cell Replication In Vivo. Diabetes 2016;65:2081-2093
55. Cui Y, Hu D, Markillie LM, Chrisler WB, Gaffrey MJ, Ansong C, Sussel L, Orr G: Fluctuation localization imaging-based fluorescence in situ hybridization (fliFISH) for accurate detection and counting of RNA copies in single cells. Nucleic Acids Res 2017;Accepted
56. Wagner DE, Klein AM: Genetic screening enters the single-cell era. Nat Methods 2017;14:237-238

## FIGURE LEGENDS

*Figure 1:* Summary of previously identified β-cell sub-populations using biomarker or single-cell analyses. *A:* Summary of functionally component ('functional') cell sub-populations including their functional and gene/protein expression profile, incidence and how they change in conditions associated with diabetes. *B:* Summary of less-functionally component ('non-functional') cell sub-populations. For *A* and *B*, those shaded are functionally defined via optogenetics in-situ. *C:* Schematic suggesting qualitative overlap between several identified sub-populations. Dashed lines indicate those defined via optogenetics in-situ. Note strong overlap between most identified larger populations, but the low incidence functional hub cell sub-population and non-functional $Ucn3^-$ sub-population show relatively lower overlap. This may however reflect the number and nature of the markers measured, as well as the functional readout. Note: i) $GFP^{high}$ increases from 10-40% with age (from 5-9w to 16-40w), $GFP^{low}$ decreases from 50% to 10% with age. ii) PSA-$NCAM^+$ is defined as top 50% expression. iii) Incidence was 50% in T2D donors. iv) Incidence was <30% in T2D donors. (v) $Ucn3^-$, $insulin^+$ located at islet periphery.

*Figure 2:* Example for how heterogeneous cells can interact. *A*: Introducing $Kir6.2^{[\Delta N30,\ K185Q]}$ into β-cells renders them non-glucose responsive. With a small (~10%) population of these non-functional cells in the islet (dark green) glucose-responsiveness is unchanged. However, with ~20% of these cells glucose responsiveness is lost. Glucose responsiveness is recovered by electrically isolating (Cx36ko) these non-functional cells. *B*: Conversely if a large proportion (~70%) of hyper-excitable $Kir6.2^{[AAA]}$ cells are introduced into the islet (light green), glucose-responsiveness remains, although this is lost when electrical coupling is lost (Cx36ko). *C*: In the native islet the number of intrinsically activated (light grey) cells increases as glucose concentration rises. However, only when a certain threshold of cells are activated, does $Ca^{2+}$ elevate as a result of insufficient numbers of non-activated cells (dark grey), analogous to *A* and *B*. *D*: The presence of activated and non-activated cells can be further revealed by the $K_{ATP}$ opener diazoxide, which renders only a small number of cells intrinsically inactive as observed in the absence of gap junction (GJ) coupling, but fully abolishes $Ca^{2+}$ elevations in the presence of GJ coupling.

*Figure 3:* Heterogeneity in the intact islet. *A:* Different β-cell sub-populations co-exist within the islet, each characterized by different gene/protein expression patterns, morphological markers, glucose-responsiveness, insulin secretion, proliferative capacity and function, as also indicated in the table in Fig.1. $Fltp^-$ proliferative (blue) and $Ucn3^-$ transdifferentiating (purple) 'immature' non-functional populations are likely non-electrically coupled and thus do not significantly affect islet-wide responses to glucose. *B:* Optogenetic mapping reveals highly functional β-cell sub-populations with varying identity markers, metabolic properties and $Ca^{2+}$ responses, but all with the ability to exert disproportionate control over coordination and intra-islet $Ca^{2+}$ responses. This includes eNpHR3.0-silenced β-cells (hub cells, green) in which halorhodopsin activation and membrane hyperpolarization disproportionally silences the islet, and ChR2-activated cells (red), in which ChR2 activation and membrane depolarization disproportionally activates the islet. A population of cells in which ChR2 activation has little effect also shows pacemaker-like characteristics owing to their higher intrinsic oscillation frequency.

A

| 'Functional' sub-populations | | | | | | |
|---|---|---|---|---|---|---|
| **Population** | **Characteristics** | **Gene and protein expression** | **Incidence** | **Proliferation** | **Maturity** | **Plasticity** |
| Mip-GFP$^{high/medium}$ | High insulin granule content | High insulin expression | ~90%(i) | Increased senescence | mature | Increased with age |
| PSA-NCAM$^{+}$ ($\beta^{high}$) | High [$Ca^{2+}$], ATP, cAMP elevation, improved insulin secretion | High *Gck*, *Glut2* expression. High exocytotic machinery levels. | ~50%(ii) | - | mature | Decrease in obesity |
| Fltp$^{+}$ | Increased oxidative metabolism, increased insulin granule content | High expression of genes for GCK, GLUT2, GLP1R, GIPR, Cx36, cellular respiration, mitochondrial organization | ~80% | Lower proliferative capacity | mature | Decrease during development, metabolic stress, |
| ST8SIA1$^{-}$ | Improved insulin secretion | High expression of *GLUT2* | ~85%(iii) | - | mature | Reduced in T2D |
| C1 (cytof) | - | High insulin expression | 10-70%(iv) | - | higher Pdx1 | Increased with age, decrease in obesity. |
| Hub | High [$Ca^{2+}$] recruitment. | High GCK, low insulin levels | 1-10% | - | Relatively immature | Disrupted by diabetogenic environment |
| ChR2$^{high}$ | High [$Ca^{2+}$] recruitment, high NAD(P)H | - | ~5% | - | - | Disrupted by diabetogenic environment |

B

| 'Non-functional' sub-populations | | | | | | |
|---|---|---|---|---|---|---|
| **Population** | **Characteristics** | **Gene and protein expression** | **Incidence** | **Proliferation** | **Maturity** | **Plasticity** |
| Mip-GFP$^{low}$ | Low insulin granule content | Low insulin expression | ~10%(i) | No senescence with age | immature | Reduced with age |
| PSA-NCAM$^{-}$ ($\beta^{low}$) | Low[$Ca^{2+}$] elevation, ATP elevation, reduced insulin secretion | Low *Gck*, *Glut2* expression | ~50%(ii) | - | Immature | Increase in obesity |
| Fltp$^{-}$ | Reduced oxidative metabolism, reduced insulin granule content | Low expression of genes for GCK, GLUT2, GLP1R, GIPR, Cx36, cellular respiration, mitochondrial organization | ~20% | Higher proliferation | immature | Increased during development, metabolic stress, |
| ST8SIA1$^{+}$ | Reduced insulin secretion | Low expression of GLUT2, higher expression K+ channels | ~15%(iii) | - | immature | Increased in T2D |
| Ucn3$^{-}$ | No glucose influx, membrane depolarization or [$Ca^{2+}$] elevation | Decreased expression of genes for GCK, TCA cycle, ox-phos; $K_{ATP}$, $Ca_V$, GLP1R, Insulin. | ~2-3%(v) | α-β transition | very immature | Increased in development |
| 'bottom' | Low insulin granule content. Resistant to immune damage | Low GLUT2, insulin expression | ~15% | Higher proliferation | Immature | Increased with T1D progression |
| ChR2$^{low}$ | Low [$Ca^{2+}$] recruitment, low NAD(P)H, wave initiation | - | ~10% | - | - | Disrupted by diabetogenic environment |

C

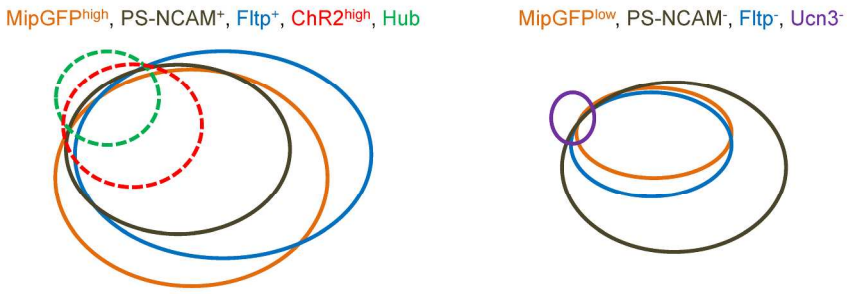


Figure 1: Summary of previously identified β-cell sub-populations using biomarker or single-cell analyses. A: Summary of functionally component ('functional') cell sub-populations including their functional and gene/protein expression profile, incidence and how they change in conditions associated with diabetes. B: Summary of less-functionally component ('non-functional') cell sub-populations. For A and B, those shaded are functionally defined via optogenetics in-situ. C: Schematic suggesting qualitative overlap between several identified sub-populations. Dashed lines indicate those defined via optogenetics in-situ. Note strong overlap between most identified larger populations, but the low incidence functional hub cell sub-population and non-functional Ucn3- sub-population show relatively lower overlap. This may however reflect the number and nature of the markers measured, as well as the functional readout. Note: i) GFPhigh increases from 10-40% with age (from 5-9w to 16-40w), GFPlow decreases from 50% to 10% with age. ii) PSA-NCAM+ is defined as top 50% expression. iii)- Incidence was 50% in T2D donors. iv) Incidence was <30% in T2D donors. (v)- Ucn3-, insulin+ located at islet periphery.

233x286mm (300 x 300 DPI)

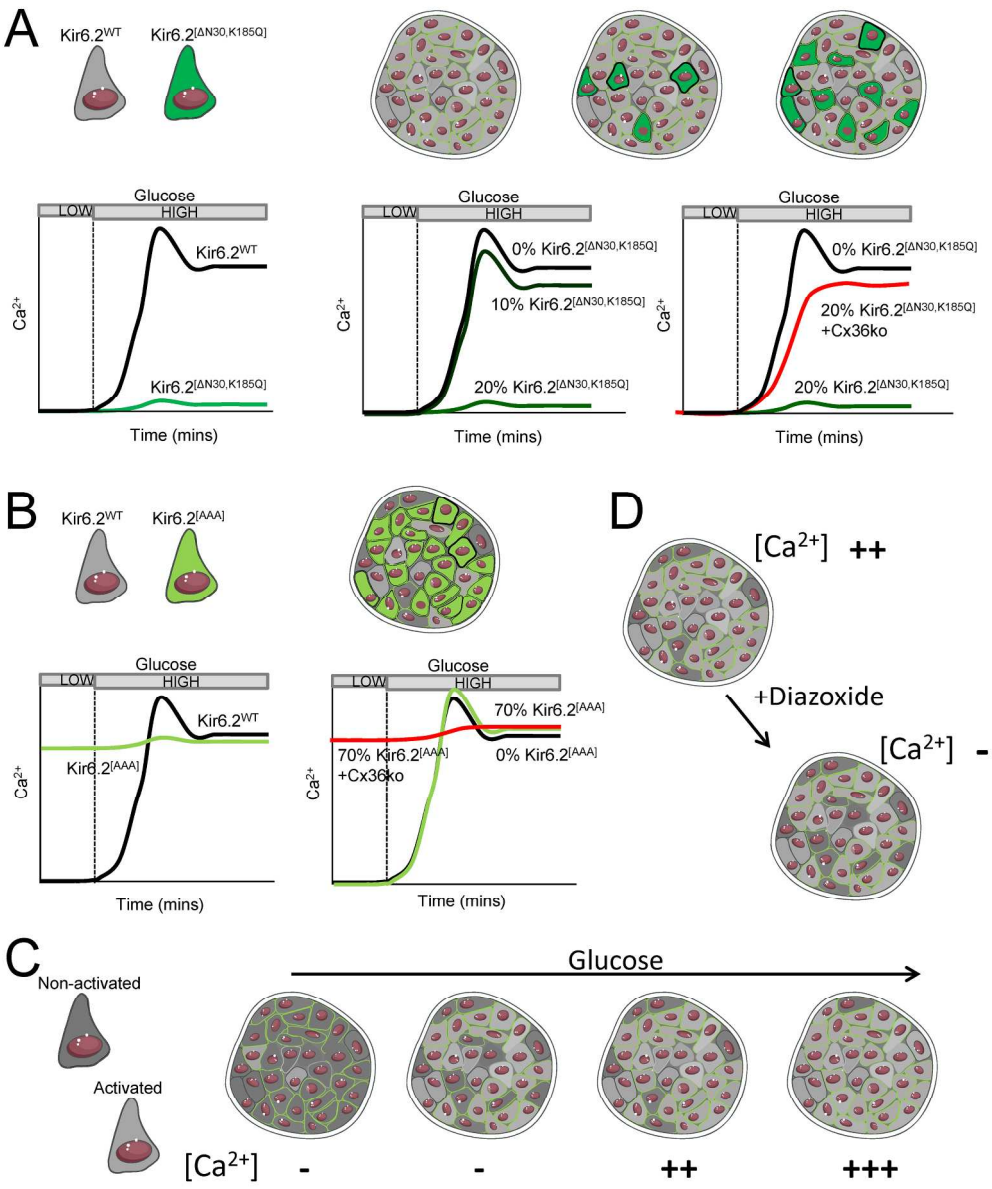


Figure 2: Example for how heterogeneous cells can interact. A: Introducing Kir6.2[ΔN30, K185Q] into β-cells renders them non-glucose responsive. With a small (~10%) population of these non-functional cells in the islet (dark green) glucose-responsiveness is unchanged. However, with ~20% of these cells glucose responsiveness is lost. Glucose responsiveness is recovered by electrically isolating (Cx36ko) these non-functional cells. B: Conversely if a large proportion (~70%) of hyper-excitable Kir6.2[AAA] cells are introduced into the islet (light green), glucose-responsiveness remains, although this is lost when electrical coupling is lost (Cx36ko). C: In the native islet the number of intrinsically activated (light grey) cells increases as glucose concentration rises. However, only when a certain threshold of cells are activated, does Ca2+ elevate as a result of insufficient numbers of non-activated cells (dark grey), analogous to A and B. D: The presence of activated and non-activated cells can be further revealed by the KATP opener diazoxide, which renders only a small number of cells intrinsically inactive as observed in the absence of gap junction (GJ) coupling, but fully abolishes Ca2+ elevations in the presence of GJ coupling.

227x272mm (300 x 300 DPI)

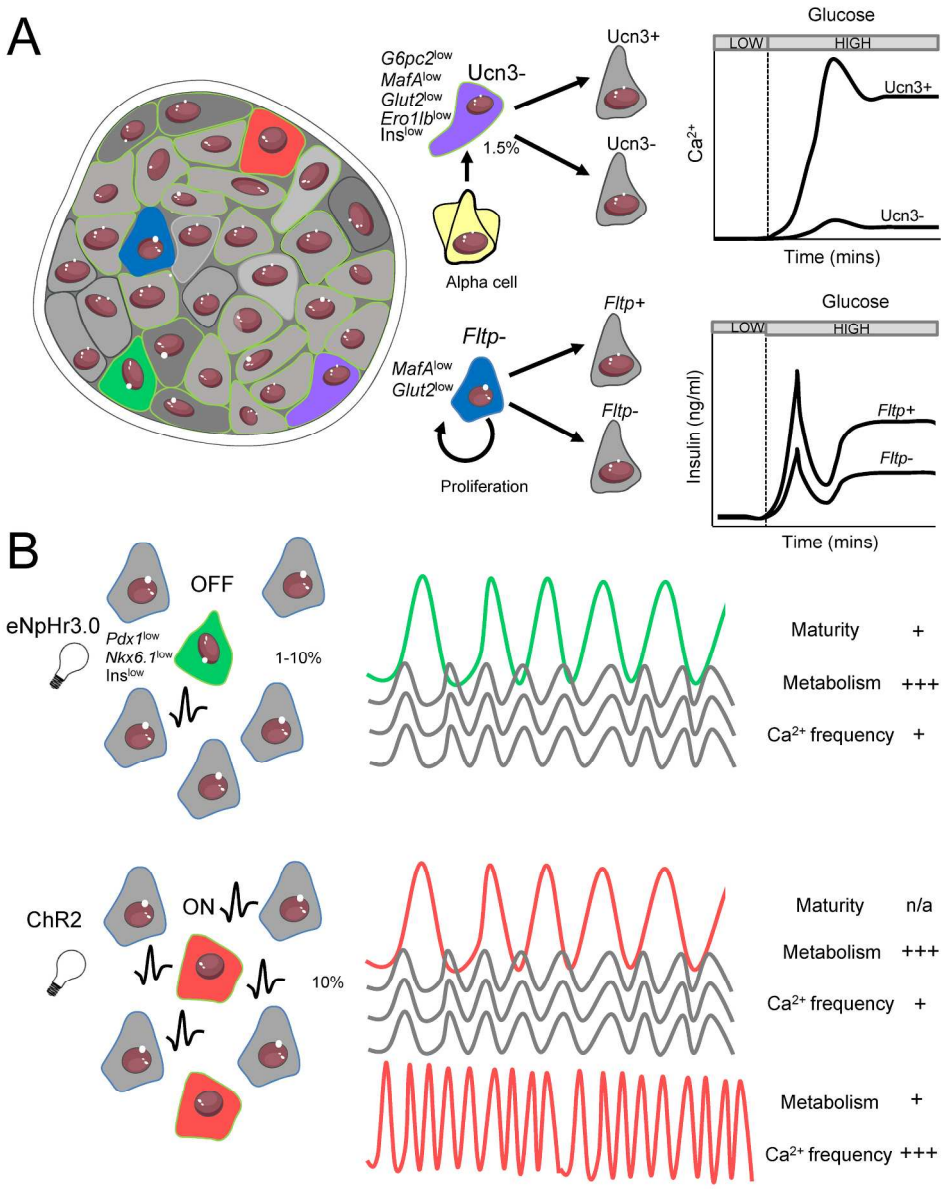


Figure 3: Heterogeneity in the intact islet. A: Different β-cell sub-populations co-exist within the islet, each characterized by different gene/protein expression patterns, morphological markers, glucose-responsiveness, insulin secretion, proliferative capacity and function, as also indicated in the table in Fig.1. Fltp- proliferative (blue) and Ucn3- transdifferentiating (purple) 'immature' non-functional populations are likely non-electrically coupled and thus do not significantly affect islet-wide responses to glucose. B: Optogenetic mapping reveals highly functional β-cell sub-populations with varying identity markers, metabolic properties and Ca2+ responses, but all with the ability to exert disproportionate control over coordination and intra-islet Ca2+ responses. This includes eNpHr3.0-silenced β-cells (hub cells, green) in which halorhodopsin activation and membrane hyperpolarization disproportionally silences the islet, and ChR2-activated cells (red), in which ChR2 activation and membrane depolarization disproportionally activates the islet. A population of cells in which ChR2 activation has little effect also shows pacemaker-like characteristics owing to their higher intrinsic oscillation frequency.

231x286mm (300 x 300 DPI)